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A COMPARATIVE STUDY: METHODS FOR THE DETERMINATION OF ASCORBIC ACID IN SMALL AND MIDDLE SIZED FOOD ANALYTIC LABORATORIES

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Although vitamin C is essential as an antioxidant and as a cofactor in a series of enzymatic reactions, the ability for ascorbate biosynthesis was lost in humans. Thus, horticultural products and derived fruit drinks or commercial vitamin C products are considered to be important sources for the ascorbic acid intake in the human diet. These facts underline the importance of analytical methods for ascorbic acid determination in different food products.

In our study two spectrophotometric and a fluorometric ascorbic acid determination methods have been compared with each other and with the so-called etalon HPLC method to find the best for small or middle sized food analytic laboratories with a sample number of up to several hundreds. As a result of our experiments we could establish that the OPDA-fluorometric method can be suggested for the determination of samples containing ascorbate at low concentrations. Unfortunately, the analytical properties of the OPDA method with spectrophotometric detection have been lagging far behind the others. The 2,2'-bipyridyl method could give a balanced performance for all tests. Furthermore, the results gained by this method are the closest to the results of the reference HPLC method in the case of fruit and vegetable samples.

Keywords: vitamin C, determination, HPLC, food, photometric, fluorometric

Vitamin C (ascorbic acid) plays an important role in the adversity of physiological reactions, including protein folding, iron metabolism, immunity and antioxidant defence (Padh, 1990; Mandl et al., 2009; Lachapelle & Drouin, 2011; Szarka & Lőrincz, 2014). Although ascorbate seems to be essential for antioxidant homeostasis and as a cofactor in a series of enzymatic reactions, the ability for ascorbate biosynthesis was lost due to mutations in the gulonolactone oxidase (GLO) gene (Nishikimi & Yagi, 1991) in the primate lineage leading to monkeys and apes (Burns 1957; Mandl et al., 2009). Hence humans are not able to synthesize ascorbic acid. Since other ascorbate-deficient species also evolved through several other lineages, it appears that the inactivation of the GLO gene occurred several times during evolution (Chaudhuri & Chatterjee, 1969; Birney et al, 1976; Dabrowski, 1990). These circumstances and the fact that the mutation did not remain a polymorphism affecting only a minority of the population, but after a positive selection mechanism only the mutant type survived, should mean that this change was advantageous. Thus, other sources have to be utilized to supply ascorbic acid to our tissues.

Horticultural products and derived fruit drinks or commercial vitamin C products are considered to be very important sources for the ascorbic acid intake in the human diet

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(Vermeir et al., 2008). According to this approach, the ascorbic acid content and the positive effect of the vitamin contribute significantly to the nutritional value of fruit and vegetables. Considering these facts, the increasing importance of analytical methods for ascorbic acid determination is not surprising.

Several analytical methods to quantify the amount of ascorbic acid in biological and food samples have been developed (Reviewed by Washko et al., 1992). One of the most simple methods is the determination of ascorbate by 2,2'-bipyridyl. In this method, determination of ascorbic acid relies on the reduction of ferric ion to ferrous ion by ascorbic acid and the quantification of the ferrous ion as the red-orange, 2,2'-bipyridyl complex. In the presence of orthophosphoric acid at pH 1–2, the reaction of 2,2'-bipyridyl with other reducing or interfering materials, e.g. reductone, glucosone, reductic acid, α-tocopherol, glutathione, cysteine, acetol, methyl glyoxal, or creatinine, are inhibited (Koenig et al., 1943; Omaye et al., 1979). The specificity of the determination can be elevated by the application of an enzyme. This can be accomplished by the oxidation of ascorbic acid by ascorbate oxidase enzyme to dehydroascorbic acid (DHA), which can be determined photometrically as a quinoxaline-derivative that formed by an interaction between DHA and *o*-phenylenediamine (OPDA). The complex can be measured at the end point of the reaction by either absorbance (Lee et al., 1997; Vermeir et al., 2008) or fluorescence detection (Wu et al., 2003; Vislisel et al., 2007). In recent years, HPLC has become the preferred method of ascorbate determination, however, this method has the disadvantages of being relatively labour intensive and costly (Lee et al., 1997).

In this study we aimed at the comparison of the two different spectrophotometric methods and a fluorometric method for vitamin C quantification: the 2,2'-bipyridyl method and the enzymatic method with two different detection modes. Their analytical properties (linearity, accuracy, precision, limit of detection, and limit of quantitation) were compared with each other and the generally accepted and previously validated (Harapanhalli et al., 1993) HPLC method. Samples were prepared from fruit and vegetable species, such as lemon (*Citrus medica*), orange (*Citrus sinensis*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*). This way we tried to test the application opportunities of the different methods and find the best for food analytical purposes.

1. Materials and methods

1.1. Materials

L(+)-Ascorbic acid, NaH_2PO_4, Na_2HPO_4, glacial acetic acid solution, absolute ethanol, and $FeCl_3$ were purchased from Reanal Finechemical Co. (Budapest, Hungary), *ortho*-phenylenediamine (OPDA), 2,2'-bipyridyl and ascorbic acid oxidase enzymes were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All the other chemicals used were of analytical grade.

The fruit and vegetable samples were purchased at the Grand Market of Budapest (Fővámtér, Budapest, Hungary).

1.2. Solutions

Five percent acetic acid solution was used for extraction of ascorbic acid from the samples.

A 100 mM stock solution of ascorbic acid was prepared by dissolving ascorbic acid crystals in 5% acetic acid solution. This stock solution was stored for 1 week at –80 °C, and remained stable during this interval. Working calibrator solutions were made prior to use by diluting the stock solution in 5% acetic acid solution to the concentrations of 1, 5, 25, 50, 100, 250, 500, 1000, 2000, 2500, and 5000 µM.

The required amount of *o*-phenylenediamine solution was made fresh every time before use by dissolving solid chemical to a concentration of 4.6 mM in 0.1 M phosphate buffer (pH 6.5), then it was stored in the dark until use.

Stock solution of ascorbic acid oxidase enzyme was prepared by dissolving the lyophilised enzyme in 0.1 M phosphate buffer to obtain an enzyme solution with an activity of 200 U ml^{-1}. 12.5 µl aliquots of this stock solution were stored at –80 °C, and diluted immediately before use with phosphate buffer to 250 µl to obtain a working solution of 10 U ml^{-1}.

The 2,2'-bipyridyl and $FeCl_3$ solutions (1%) were prepared fresh before use by dissolving the crystals of the chemical in absolute ethanol.

1.3. Sample preparation

All the sample extracts were prepared by slicing the fruit or vegetable into small pieces and immediately freezing in liquid nitrogen. Thereafter, the samples were smashed using a porcelain mortar and pestle, then known amounts of the frozen tissue samples were homogenised in 5% acetic acid solution. The volumes of the homogenates were recorded and the samples were centrifuged at 16 500 *g* for 5 min at 4 °C. The supernatants of the extracts were collected and stored at –80 °C until use.

1.4. 2,2'-bipyridyl method

75 µl 85% orthophosphoric acid, 750 µl 1% 2,2'-bipyridyl, and 300 µl 1% $FeCl_3$ solutions were added to 300 µl of samples in a microcentrifuge tube.

The samples were incubated for 60 min at room temperature, then they were centrifuged at 16 500 *g* for 5 min. Into a well of a microtiter plate 350 µl of the supernatant was transferred. Each sample were analysed in three replicates. The absorbance values were read at 525 nm by a Thermo Multiskan Go plate reader.

Blanks contained all the reagents for the assay but instead of ascorbate phosphate buffer was added. All samples had a control-pair to determine the background of the samples. For control measures absolute ethanol instead of 2,2'-bipyridyl solution was added to the reaction.

1.5. Enzymatic methods (OPDA, OPDA-fluorometric)

Three hundred microlitres of OPDA solution (4.6 mM in 0.1 M phosphate buffer) and 15 µl ascorbic acid oxidase enzyme solution (10 U ml^{-1} in 0.1 M phosphate buffer) were added to 37.5 µl sample, control, or standard solution in a microtiter plate well. The absorbance has been measured at 340 nm after shaking and 10 min of incubation at room temperature.

Alternatively, fluorescence was measured (Jasco FP-8200 spectrofluorometer) using 340 nm for excitation and 420 nm for emission.

Blanks were prepared by using phosphate buffer instead of the solution containing ascorbic acid. Control solutions contained all the reagents for the assay but substituting phosphate buffer for the fraction of ascorbic acid oxidase enzyme solution.

All standards, samples, and controls were measured by both detection modes in three replicates.

1.6. The reference HPLC method

The isocratic reverse phase HPLC analysis was carried out with a Waters Series 2690 Separations Module and Waters Model 2487 dual absorbance UV detector at 254 nm. The separations were carried out on a Teknokroma TR-011349 NUCLEOSIL 100 C18 column (average particle size 5 μm, 25 cm × 0.46 mm). Mobile phase was 0.1 M NaH_2PO_4 and 0.2 mM Na_2EDTA, adjusted to pH 3.1 with orthophosphoric acid (Szarka et al., 2002; Zsigmond et al., 2011).

2. Results and discussion

The most objective way to compare different analytical methods is the determination of their analytical properties such as the linearity, accuracy, precision, limit of detection and limit of quantitation. Thus, all these parameters were determined for the 2,2'-bipyridyl and the ascorbate oxidase - OPDA method with two different detection modes. The spectrophotometric and fluorometric methods were compared with each other and with the so-called etalon HPLC method (Washko et al., 1992).

2.1. Linearity

The comparison of measurements by two analytical methods is usually based on a form of regression analysis (RA). The linearity of different methods was determined by the following ascorbic acid standards prepared in 5% acetic acid solution: 0, 1, 5, 25, 50, 100, 250, 500, 1000, 2000, 2500, 5000, and 10 000 μM. The linear ranges, the equations of linear regression, and the coefficient of determination (R^2) values were calculated (Table 1). The calibration curves were considered to be linear, if R^2>0.98. However, it should be noticed that the most frequently used approach, least-squares regression analysis, is subject to several shortcomings when both measurement sets are subject to random errors, which represents the typical situation. Therefore, alternative regression procedures are more appropriate to apply. The Deming method takes measurement errors for both sets of measurements into account and is therefore more generally applicable than least-squares regression analysis (Linnet, 1998). Thus, the weighted-Deming regression analysis has also been applied for the comparison of 2,2'-bipyridyl and OPDA methods to the reference (HPLC) method (Table 1).

The OPDA-fluorometric method has shown the highest sensitivity among the three tested methods. However, it should also be noticed that its value is still remained far behind the sensitivity of chromatographic technique (Table 1). Furthermore, this method can be characterized by the narrowest linear range (0–250 μM), however, the relatively low upper limit should not cause a problem, because concentrated samples can easily be diluted to get within the linear range of the assay. This narrow linear range could be easily seen by the survey of the slope values gained by the Deming RA (Table 1). (The slope of Deming RA has also been determined out of the linear range of OPDA-fluorometric method, but within the

linear range of the reference HPLC method (Table 1).) The 2,2'-bipyridyl and the OPDA method can be used in a wider range, the linear range of 2,2'-bipyridyl method was limited only by the performance of the spectrophotometer that was used. Furthermore, this method could be characterized by the best Deming RA slope value (Table 1).

Table 1. Comparison of the linearity of different methods for ascorbate determination

Method	Linear range (μM)	Least-squares RA (slope)	Weighted-Deming RA (slope)
Reference (HPLC)	0.5–1000[#]	0.9995	
2,2'-bipyridyl	5–1000	0.9995	1.009
OPDA	22–5000	0.9975	1.055
OPDA fluoro.	0.5–250	0.9980	1.036
OPDA fluoro.	0.5–1000*	0.9198	0.892

[#]: The linearity of HPLC method has not been investigated for wider range.*: Out of the linear range of OPDA fluoro., but within the linear range of the reference (HPLC) method.

2.2. Accuracy

The accuracy is defined as the percent deviation between the measured and the nominal values. The 2,2'-bipyridyl and the OPDA method have similar accuracy in the higher concentration range, however, both methods (especially the 2,2'-bipyridyl) have shown significantly higher percent deviation at lower concentrations (Table 2). It is not surprising, since both methods can be characterized by quite high LoD, LoQ (low sensitivity) (chapter *2.4*) (McGown et al., 1982). The OPDA-fluorometric method has an intermediate accuracy in its linear range (Table 2) as it could be expected on the base of its higher sensitivity (chapter *2.4*). Surprisingly, at higher ascorbate concentrations the accuracy of all the three tested methods could approximate or exceed the accuracy of the reference HPLC method (Table 2). The HPLC method can perform better only at low concentrations, which can be easily avoided in the case of fruit and vegetable samples.

Table 2. The accuracy of different methods for ascorbate determination

Concentration (μM)	Accuracy (%)			
	HPLC	2,2'-bipyridyl	OPDA	OPDA fluoro.
5.00	13.46	30.73	23.73	2.57
50.00	3.60	0.53	1.80	0.03
500.00	7.67	1.06	0.48	–
1000.00	1.93	0.31	0.11	–
Mean	8.25	8.16	6.53	1.30

The concentrations were selected from the linear range of different methods. All results are presented as the averages of three independent measurements.

2.3. Precision

Precision is defined as the deviation between the results of different measurements of the same homogeneous sample. Standard deviation (SD) values are used to characterize the precision. The repeatability of the measurements was investigated by using the same instrument within a short period. The repeatability of the OPDA-fluorometric method (within the linear range) exceeded the other investigated methods by far (Table 3). Both OPDA methods showed perfect precision compared with the HPLC method. However, the 2,2'-bipyridyl method showed elevated SD values, especially at higher concentrations (Table 3).

Table 3. The repeatability of different methods for ascorbate determination

Concentration (μM)	Precision (SD)			
	HPLC	2,2'-bipyridyl	OPDA	OPDA fluoro.
5.00	0.33	4.07	0.79	0.02
50.00	2.40	1.39	3.59	0.00
500.00	5.07	21.88	4.55	–
1000.00	15.83	11.35	2.05	–
Mean	5.91	9.67	2.74	0.01

The investigated concentrations were selected from the linear ranges of different methods. All results are presented as the averages of three independent determinations.

2.4. Limit of detection, limit of quantification

Limit of detection (LoD) and limit of quantification (LoQ) are terms used to describe the lowest concentration of an analyte that can be reliably determined or quantified by an analytical procedure. Both analytical properties were calculated based on the standard deviation of background's (blank) response and the slope of the calibration curve:

$$\mathrm{LoD} = \frac{(3 \times \mathrm{SD_b})}{\mathrm{S}},$$

$$\mathrm{LoQ} = \frac{(10 \times \mathrm{SD_b})}{\mathrm{S}},$$

where SD_b=Standard deviation of blank's response, S=Slope of the calibration curve.

The LoD and LoQ values for the OPDA-fluorometric and the reference HPLC methods are almost the same (Table 4). As it was expected from the earlier observations (McGown et al., 1982), the 2,2'-bipyridyl method could not reach the LoD and LoQ values of HPLC method. The OPDA method showed clearly the highest LoD and LoQ values, thus, the application of this method cannot be advised at concentrations lower than 7 μM.

Table 4. The LoD and LoQ values of different methods for ascorbate determination

Method	LoD (μM)	LoQ (μM)
Reference (HPLC)	0.11	0.38
2,2'-bipyridyl	1.34	4.46
OPDA	6.53	21.75
OPDA fluoro.	0.13	0.42

2.5. Determination of ascorbate from different fruit and vegetable samples

Our final goal was to apply and compare all the tested methods for the determination of different fruit and vegetable species found on our table. Four popular natural sources of vitamin C have been chosen, such as lemon, orange, pepper, and tomato. The vitamin C content of them all has been determined by all four methods (Table 5). Similar tendency can be drawn in all cases. The results gained by the 2,2'-bipyridyl method are the closest to the results of the reference HPLC method. The ascorbic acid contents determined by the 2,2'-bipyridyl method are a bit higher than the ascorbic acid contents determined by the HPLC method in the case of orange, lemon, and pepper. However, the ascorbic acid contents determined by the 2,2'-bipyridyl method are a bit lower than the ascorbic acid contents determined by the HPLC method in the case of tomato. The differences can be explained by at least two reasons. On one hand, the 2,2'-bipyridyl method lacks the specificity due to interference from creatinine, acetol, α-tocopherol, glutathione, and cysteine, as well as other reducing substances (Washko et al., 1992). Necessarily we tried to diminish the interference by the application of orthophosphoric acid, but the interfering effects of other reducing agents of the fruit could not be ruled out. On the other hand, it should be noticed that dehydroascorbic acid cannot be determined (directly) by the 2,2'-bipyridyl method (Omaye et al., 1979). It has only limited significance, since more than 95% of vitamin C can be found in its reduced state (ascorbic acid) in plant tissues (Zsigmond et al., 2011). However, it can cause a little underestimation of vitamin C content if it has been determined by the 2,2'-bipyridyl method.

Table 5. Results of ascorbic acid determination by the tested methods

Sample	Ascorbic acid content (mg AA/100 g sample)			
	Reference (HPLC)	2,2'-bipyridyl	OPDA	OPDA fluoro.
Lemon	39.8	40.7	34.7	28.9
Orange	28.9	31.0	26.4	24.5
Pepper	146.1	148.1	133.5	126.9
Tomato	17.9	16.4	11.9	13.9

The results are corrected with controls. SD values have not exceeded the 1% in the case of all samples

This specificity issue can be avoided by the application of ascorbic acid oxidase enzyme (EC 1.10.3.3), which is highly specific for oxidizing ascorbic acid to dehydroascorbic acid (Lee et al., 1997; Vermeir et al., 2008). Thus, the OPDA methods are considered to be as specific as the reference HPLC method(s). Even so, the ascorbic acid contents determined by the OPDA and OPDA fluorimetric methods are approximately 10% (or more) lower than the

ascorbic acid contents determined by the HPLC method. It can be explained by the derivatization of ascorbic acid. When a sample containing both ascorbic acid and dehydroascorbic acid is combined with OPDA, dehydroascorbic acid is depleted by the reaction. Because the equilibrium between ascorbic acid and dehydroascorbic acid is perturbed, more dehydroascorbic acid might form, which could then react with OPDA to form more quinoxaline derivatives. The end result may be a falsely elevated value for dehydroascorbic acid and a falsely decreased value for ascorbic acid (WASHKO et al., 1992).

2.6. Throughput of the assays

The throughput of an assay can be one of its most important features. We think these ascorbic acid determination procedures can mostly be applied in small or middle sized food analytic laboratories with a sample number of up to several hundreds.

The throughput of both the 2,2'-bipyridyl and the OPDA methods could be significantly increased by detecting the absorbance or the fluorescence with a plate reader. This way the ascorbate content of 32 different samples (in the case of a 96 well plate) could be measured in three replicates within 5 min of instrument time. There is a 60 min incubation step in the 2,2'-bipyridyl method, thus, it extends the duration of this ascorbate determination process. However, there is just 10 min incubation in the OPDA methods, furthermore the OPDA-fluorometric method can also be detected by a fluorometric plate reader. Although the HPLC method is considered to be the reference method, the analysis time is 10–15 min for each sample. Hence, it is not really suitable for high throughput vitamin C measurements.

3. Conclusions

Two spectrophotometric and a fluorometric ascorbic acid determination methods have been compared with each other and with the so-called etalon HPLC method to find the best for small or middle sized food analytic laboratories with a sample number of up to several hundreds.

The OPDA-fluorometric method can be suggested for the determination of samples containing the ascorbate at low concentrations because of its low LoD and LoQ values. However, this method can be characterized by the narrowest linear range. Furthermore, the ascorbate contents of different fruit and vegetable samples have been slightly underestimated by this method, probably due to the derivatization of ascorbate. Unfortunately, the analytical properties of the OPDA method with spectrophotometric detection have been lagging far behind the others. Hence, we cannot recommend the application of this method. The 2,2'-bipyridyl method could give a balanced performance for all tests. Furthermore, the results gained by the 2,2'-bipyridyl method are the closest to the results of the reference HPLC method in the case of fruit and vegetable samples. The specificity issue of the method can be diminished by the application of orthophosphoric acid. Similarly to the OPDA-fluorometric method, the throughput of the 2,2'-bipyridyl method could be significantly increased by detecting the absorbance or the fluorescence with a plate reader.

Considering these facts, the 2,2'-bipyridyl method seems to be the most suitable method for vitamin C analysis in small or middle sized food analytic laboratories with a sample number of up to several hundreds.

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References

Birney, E.C., Jenness, R. & Ayaz, K.M. (1976): Inability of bats to synthesise L-ascorbic acid. *Nature*, *260*(5552), 626–628.

Burns, J.J. (1957): Missing step in man, monkey and guinea pig required for the biosynthesis of L-ascorbic acid. *Nature*, *180*(4585), 553.

Chaudhuri, C.R. & Chatterjee, I.B. (1969): L-ascorbic acid synthesis in birds: phylogenetic trend. *Science, 164*(3878), 435–436.

Dabrowski, K. (1990): Gulonolactone oxidase is missing in teleost fish. The direct spectrophotometric assay. *Biol. Chem. H-S., 371*, 207–214.

Harapanhalli, R.S., Howell, R.W. & Rao, D.V. (1993): Testicular and plasma ascorbic acid levels in mice following dietary intake: a high-performance liquid chromatographic analysis. *J. Chromatogr., 614*, 233–243.

Koenig, R., Schiefelbusch, T. & Johnson, C. (1943): Chromogenic reagent for vitamin C determinations. *Ind. Eng. Chem.*, *15*(3), 181–182.

Lachapelle, M.Y. & Drouin, G. (2011): Inactivation dates of the human and guinea pig vitamin C genes. *Genetica, 139*, 199–207.

Lee, W., Roberts, S.M. & Labbe, R.F. (1997): Ascorbic acid determination with an automated enzymatic procedure. *Clin. Chem., 43*, 154–157.

Linnet, K. (1998): Performance of Deming regression analysis in case of misspecified analytical error ratio in method comparison studies. *Clin. Chem., 44*, 1024–1031.

Mandl, J., Szarka, A. & Bánhegyi, G. (2009): Vitamin C: update on physiology and pharmacology. *Br. J. Pharmacol., 157*, 1097–1110.

McGown, E.L., Rusnak, M.G., Lewis, C.M. & Tillotson, J.A. (1982): Tissue ascorbic acid analysis using ferrozine compared with the dinitrophenylhydrazine method. *Anal. Biochem., 119*, 55–61.

Nishikimi, M. & Yagi, K. (1991): Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. *Am. J. Clin. Nutr., 54*(6 Suppl), 1203S–1208S.

Omaye, S.T., Turnbull, J.D. & Sauberlich, H.E. (1979): Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. *Method. Enzymol., 62,* 3–11.

Padh, H. (1990): Cellular functions of ascorbic acid. *Biochem. Cell Biol., 68,* 1166–1173.

Szarka, A., Stadler, K., Jenei, V., Margittai, E., Csala, M., Jakus, J., Mandl, J. & Bánhegyi, G. (2002): Ascorbyl free radical and dehydroascorbate formation in rat liver endoplasmic reticulum. *J. Bioenerg. Biomembr., 34*, 317–323.

Szarka, A. & Lőrincz, T. (2014): The role of ascorbate in protein folding. *Protoplasma, 251*, 489–497.

Vermeir, S., Hertog, M.L.A.T.M., Schenk, A., Beullens, K., Nicolaï, B.M. & Lammertyn, J. (2008): Evaluation and optimization of high-throughput enzymatic assays for fast L-ascorbic acid quantification in fruit and vegetables. *Anal. Chim. Acta, 618*, 94–101.

Vislisel, J.M., Schafer, F.Q. & Buettner, G.R. (2007): A simple and sensitive assay for ascorbate using a plate reader. *Anal. Biochem., 365*, 31–39.

Washko, P.W., Welch, R.W., Dhariwal, K.R., Wang, Y. & Levine, M. (1992): Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Anal. Biochem., 204*, 1–14.

Wu, X., Diao, Y., Sun, C., Yang, J., Wang, Y. & Sun, S. (2003): Fluorimetric determination of ascorbic acid with o-phenylenediamine. *Talanta, 59*, 95–99.

Zsigmond, L., Tomasskovics, B., Deák, V., Rigó, G., Szabados, L., Bánhegyi, G. & Szarka, A. (2011): Enhanced activity of galactono-1,4-lactone dehydrogenase and ascorbate-glutathione cycle in mitochondria from complex III deficient *Arabidopsis. Plant Physiol. Bioch., 49*, 809–815.